Short-term and long-term effects of optical clearing agents on blood vessels in chick chorioallantoic membrane

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Abstract. The tissue optical clearing technique shows great potential in optical diagnosis and therapy. However, short-term and long-term effects of optical clearing agents on blood vessels, which are relevant to the safety of clinical applications, have not been clarified. We used laser speckle contrast imaging to monitor the changes in blood vessels in chick chorioallantoic membrane (CAM) after application of glycerol or glucose. The changes in morphology of vessels and blood flow velocity were measured. Long-term effects on blood vessels were investigated by observing the function and the development of blood vessels. The results show that glycerol reduces the local blood flow velocity and constricts and even blocks vessels quickly. At 2 days, the blood flow velocity is recovered to different extents, and new blood vessels develop but are fewer in number. Glucose induces slow changes in blood flow or vessels. However, most blood vessels are blocked, and no new blood vessel develops at 2 days. The effects depend on the dosage of agents, including volume and concentration, and decrease with the dosage of agents. Therefore, short-term effects of glucose on blood vessels are slighter than those of glycerol, but long-term effects of glucose are greater. [DOI: 10.1117/1.2907169]

Keywords: laser speckle contrast imaging (LSCI); chick chorioallantoic membrane (CAM); blood flow; vessel diameter; optical clearing agents; short-term effects; long-term effects.

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1 Introduction

Optical technology for medical diagnosis and therapy has been a focus of medicine and engineering, but the limited penetration of visible and near-infrared light caused by the high scattering in biological tissues affects its application in clinical medicine. The tissue optical clearing technique based on immersion of tissues into optical clearing agents, proposed by Tuchin,[1–4] can improve the depth to which light penetrates, and currently attracts great attention.[1–25]This technique, combined with other optical imaging techniques, such as optical coherence tomography (OCT),[2,3,14] fluorescence imaging,[5,6] microscopy imaging,[7,8] and laser radiation,[9,10] etc. will enhance the capabilities of noninvasive optical diagnosis and therapeutic treatments.

Recently, much work has been conducted in this field.[1–25]Most investigators paid attention to physical[11–13] or chemical[14–18] ways to enhance the penetration depth of light and explored the mechanism of optical clearing.[19–22] Glycerol and glucose, the common agents, have been proved to be effective for the optical clearance of tissue.[1–3,5] However, some side effects of the two agents on blood vessels have been found.[23–25] Cheng et al.[23] noticed that 100% glycerol induced a decrease of cerebral blood flow and indicated that it is not a good choice for *in vivo* clearing although it is not toxic. Vargas et al.[24] observed that 100% and 75% glycerol acting on the subdermis of hamster blocked blood flow in the venules. Galanzha et al.[25] also found blockage of microvessels to different extents after 75% glycerol or 20% to 40% glucose were added to the mesentery of rat. Accordingly, before the optical clearing technique is applied to clinical medicine, it is most important that the optical clearing agents should be not only effective, but also safe for humans.

Among the investigations on effects of optical clearing agents on blood vessels, Cheng et al.[23] noticed that glycerol induced change in blood flow velocity but did not show the changes in vascularity or blood diameter that are relevant to blood vessel functions. Vargas et al.[24] and Galanzha et al.[25] focused on a qualitative description rather than a quantitatively analysis. In addition, the current investigations were mainly restricted to short-term effects of optical clearing agents on blood vessels.[23–25] In reality, short-term effects might disappear or continue or even change as time goes on. Since clinical applications last much longer than these short-

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term experiments, further long-term observations are necessary to ensure safety during clinical applications of optical clearing agents.

The purpose of this paper is to inquire into short-term and long-term influences of optical clearing agents on blood vessels and to evaluate the effects of different dosages. An experimental model, the chick chorioallantoic membrane (CAM), was used. Laser speckle contrast imaging (LSCI) was applied to monitor short-term effects of glycerol or glucose at different concentrations or volumes on blood vessels, including their morphology (diameter and vascularity) and blood flow velocity. In addition, long-term effects of the agents were investigated by observing the function and development of blood vessels in CAM.

2 Material and Methods

2.1 *Preparation of the Chick Chorioallantoic Membrane*

We observed short-term and long-term effects of optical clearing agents on blood vessels in the CAM. The CAM has been used for studying biological processes such as implantation and ensuing angiogenesis,[26] and it presents an attractive alternative to whole animals in studying cancer treatments, such as the effects of photodynamic therapy on the tumor-bearing CAM.[27–29]

Fertilized eggs (55 to 65 g) were obtained from the Hubei Province Center of Laboratory Animals (Wuhan, China). They were washed with 70% alcohol and placed in an incubator (38°C in 60% humidity). Each egg was set at an angle of 45 deg from the vertical, with the broad apex upward, and turned 180 deg twice daily. On day 3, the shell of the egg was cut and broken off with fingers and thumb. The egg minus shell was placed in an asepsis cup and covered with a transparent plastic film. On the following days, the egg was placed in a stationary incubator until it had developed and was ready for experimentation. By days 6 to 9 of embryo age (EA 6 to 9), the CAM contains a dense capillary plexus.

2.2 *Optical Clearing Agents*

Among the investigations of various optical clearing agents of tissues, glycerol and glucose solutions are used commonly because they can cause significant increase of translucence of tissue.[1–3,5] Higher-concentrated glycerol (75%, 100%) and glucose (40%) have great optical clearance of tissue, but the less-concentrated glucose solutions (35% to 20%, 10 μL) have slight clearance.[24,25] Moreover, the agents have also been applied to study short-term effects on blood vessels.[23–25]

To investigate short-term and long-term effects, we used 10 μL and 40 μL of 100%, 50%, and 25% glycerol and 40% and 20% glucose in this work. The same volumes of phosphate-buffered saline were applied as the control. The 55 chick embryos were divided into 12 subgroups. This gave 4 to 5 replicates, per subgroup, for each experimental treatment.

2.3 *Experimental Setup and Protocol*

Figure 1 shows the experimental setup.[30,31] An He-Ne laser beam (λ=632.8 nm, 10 mW, Melles Griot, Carlsbad) was coupled to an 8-mm-diam fiber, which was adjusted to illuminate the area of interest evenly. The illuminated area was imaged through a zoom stereomicroscope (SZ6045TR, Olympus, Japan) onto a CCD camera (CoolSNAPes, Roperscientific, Tucson, Arizon) with 640×480 pixels, yielding an image of 0.8 mm to 7 mm. In this experiment, the area of the image is 2.4 mm×3.2 mm. The CCD exposure time T was set at 20 ms. Images were acquired through the PVCAM software (Roperscientific, Tucson, Arizona) at 40 Hz.

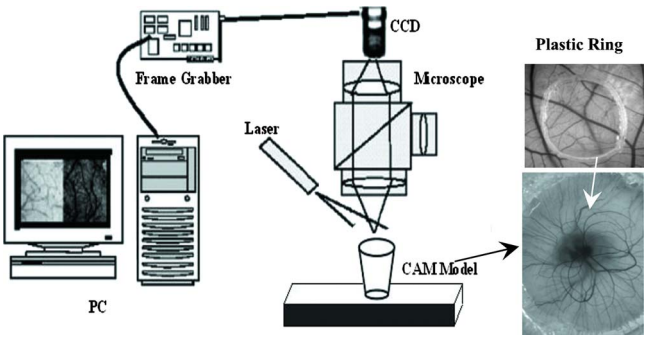

Fig. 1 Description of experimental setup. On the right is a photograph of the CAM model; the circle is the region of interest. On the left is the schematic illustration of the LSCI system.

The chick embryo (EA 6 to 7) from the incubator was placed on a thermostabilizing microscope stage (38°C). To observe the blood vessels, a plastic ring, 8 mg in weight and 10 mm in diameter, was placed around a region of interest in the CAM (see Fig. 1). A photograph and a set of raw speckle maps of a normal CAM were acquired first as a reference. Then 40 μL (or 10 μL) of warm agent (38°C) was dropped into the region. The first 9 sets of raw speckle maps were recorded at 10-s intervals, and the next 10 were recorded at 3-min intervals. In order to investigate long-term effects of the agents on blood vessels, the chick embryo was put back into the incubator after the preceding measurements. A photograph and a set of raw speckle maps of the CAM were measured again 2 days later (EA 8 to 9).

2.4 *Data Analysis*

Laser speckle is an interference pattern produced by light reflected or scattered from different parts of an illuminated surface.[32] When the area illuminated by a laser is imaged onto a CCD, a granular or speckle pattern is produced. If the scattered particles are moving, a time-varying speckle pattern will be generated at each pixel in the image. The spatial and the temporal intensity variation of this pattern contains the information about the scattered particles. The LSCI technique gives the two-dimensional (2-D) blood flow distribution with a high spatial and temporal resolution through analyzing the spatial blurring of the speckle image obtained by CCD. This blurring is represented as the local speckle contrast[33]:

$$C = \frac{\sigma_s}{\langle I \rangle} = \left\{ \frac{\tau_c}{2T} \left[1 - \exp\left(\frac{-2T}{\tau_c} \right) \right] \right\}^{\frac{1}{2}}, \qquad (1)$$

where C, σ_s, and $\langle I \rangle$ represent speckle contrast, the standard deviation, and the mean value of light intensity, respectively. τ_c is the correlation time, and T is the exposure time of the CCD. The simplest model leads to the characteristic velocity v_c[34]:

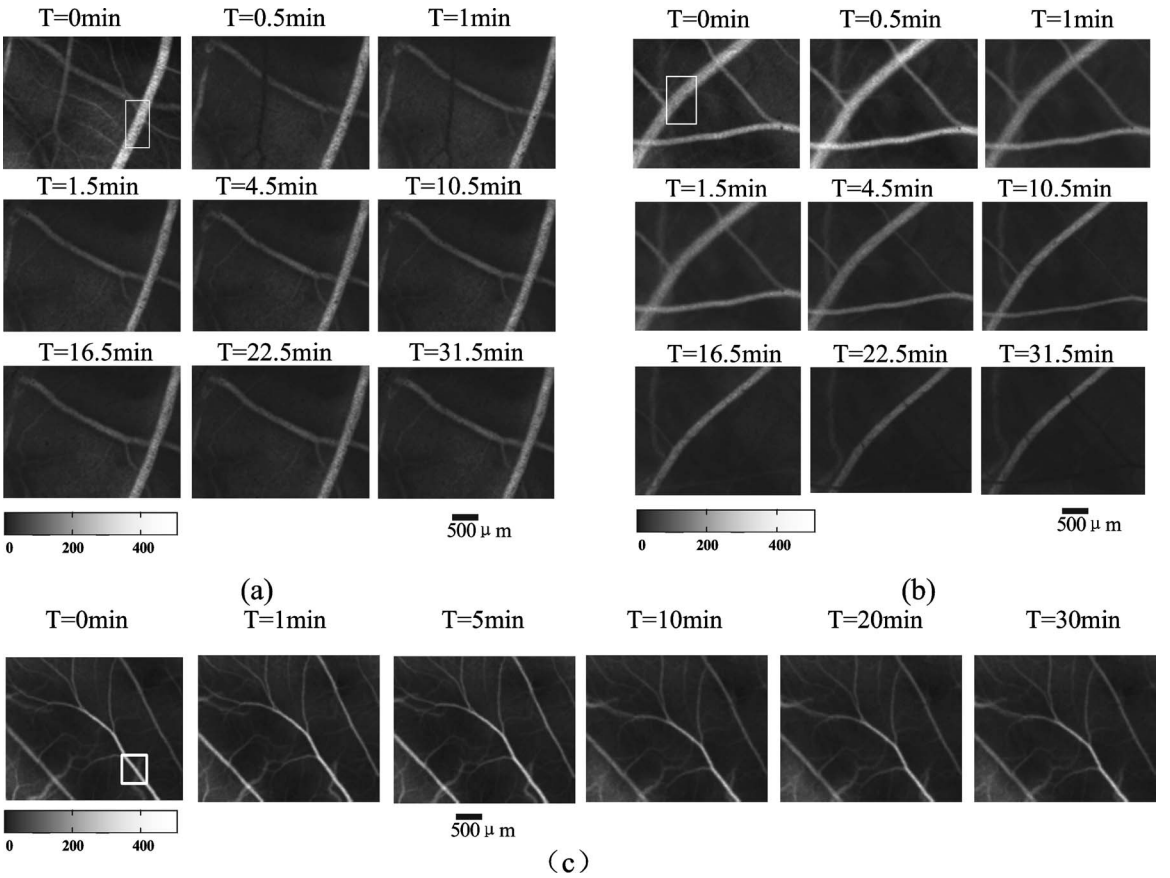

Fig. 2 A set of typical speckle velocity maps of blood vessels in CAM at 0 to 31.5 min after application of different agents (40 μL): (a) 25% glycerol, (b) 20% glucose, and (c) physiological saline. The gray scales below the images show the contrast scales, and the bar marks 500 μm.

$$v_c = \lambda/2\pi\tau_c, \tag{2}$$

where λ is the wavelength of the laser.

Since the question about absolute velocity is far from settled,[35–37] and relative measurements are all that are necessary in a clinical situation, it is more important to measure changes or differences in blood flow than to make absolute measurements.[31] Therefore, relative blood flow was obtained as follows.

Based on the raw speckle maps, we calculated the speckle contrast (C) for any given square (i.e., 5×5 pixels) and assigned this value to the central pixel of the square. This process was then repeated to obtain a set of speckle contrast maps. A corresponding set of velocity maps ($2\,T/\tau_c$) before and after the treatment was obtained from each pixel in the speckle contrast maps by the use of Eq. (1). The relative velocity in a particular vessel was calculated from the ratio of the velocity after application to the initial value.

To calculate the relative diameter of vessels, we first set a gray value in a region of interest of the velocity image as a threshold and then identified the vessels as having pixels with gray values above the threshold. Since each pixel is the same size, we can calculate the diameter of the vessel by counting the number of pixels. The mean values of the diameter were computed at each time point. The relative diameter in a vessel of interest was expressed as the ratio of the measured diameter under conditions of treatment with agents to that of the initial condition. Here we calculated values only for lumen of vessels through which blood flows. When the blood vessel was blocked, we treated the diameter of lumen of the vessel as zero.

3 Results and Analysis

3.1 *Short-Term Effects of Glycerol and Glucose on Blood Vessels*

Figures 2(a) to 2(c) show the typical velocity maps at different times after application of 25% glycerol, 20% glucose, and physiological saline, respectively. The volume of each agent given is 40 μL. The gray scale below the images indicates the contrast scales, and the bar is 500 μm. The brighter the region, the faster the blood flow, and the wider the bright region, the larger the diameter of lumen of the vessel.

The results indicate that there are different responses to the agents in the main blood vessels and their branches. Both of the optical clearing agents reduce the velocity of flow of main vessels and even block vessel branches. The effects of 25% glycerol on blood flow or vessel diameter appear more quickly than those of 20% glucose, but the constriction of the main vessels caused by 25% glycerol is less than that caused by 20% glucose. The same volume of physiological saline does not change the flow velocity or diameter of blood vessels in CAM.

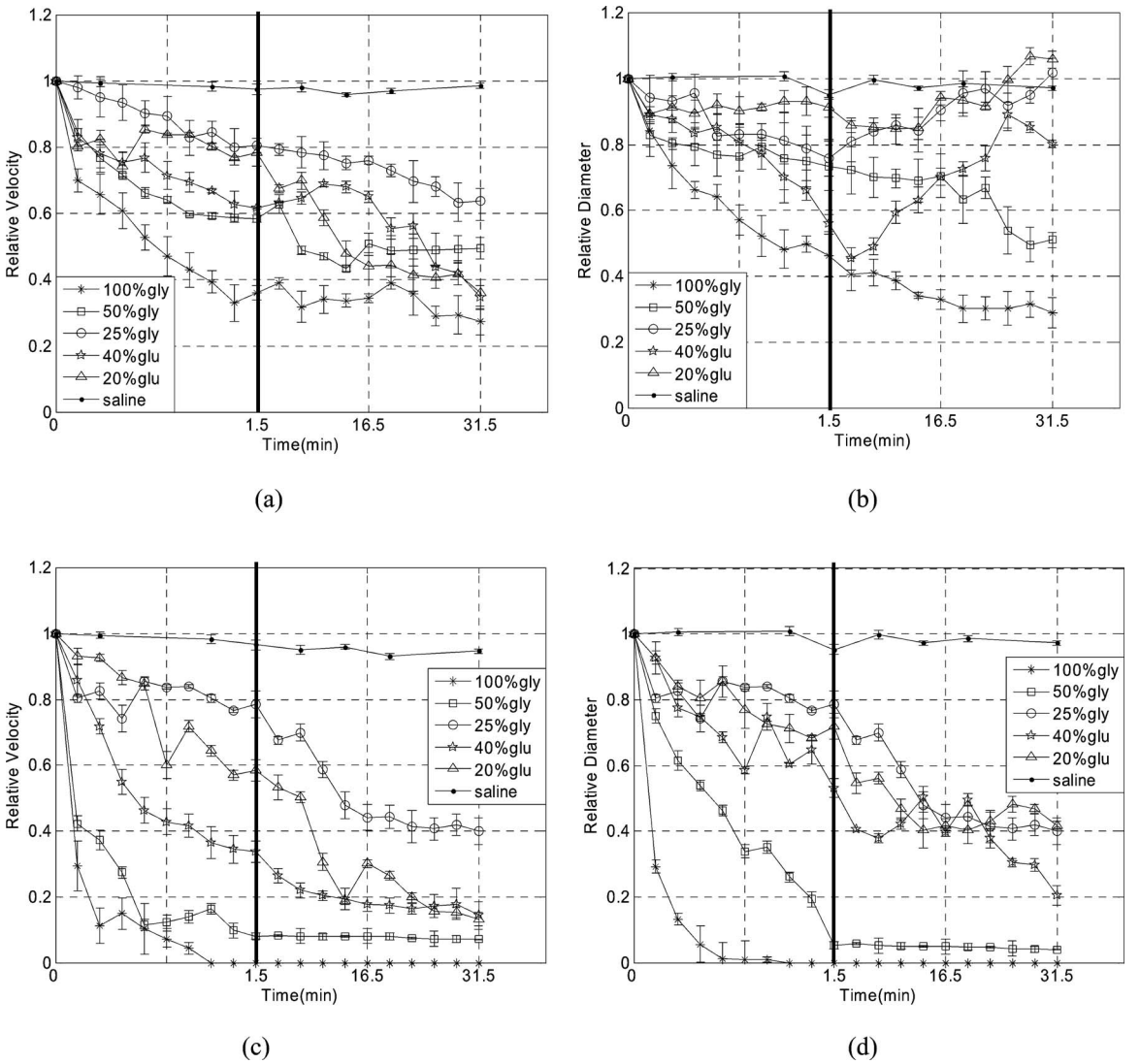

Fig. 3 The dynamic changes in the main vessels in CAM at 0 to 31.5 min after application of different agents. (a) Relative blood flow (10 μL), (b) relative diameter (10 μL), (c) relative blood flow (40 μL), and (d) relative diameter (40 μL). Each figure contains the data from six subgroups: 100%, 50%, and 25% glycerol; 40% and 20% glucose; and physiological saline. The scale of time is asymmetric. The first 9 data points on the left were recorded at 10-s intervals (0 to 1.5 min), and the next 10 data points on the right were recorded at 3-min intervals (1.5 to 31.5 min).

In order to investigate the quantitative changes in flow velocity and diameter of vessels, we focused on the 150 to 200-μm main vessels because some smaller vessel branches were blocked quickly after application of optical clearing agents. Figures 3(a) to 3(d) denote how the relative flow velocity and vessel diameter varied with time after application of 10 μL or 40 μL of glycerol or glucose at different concentrations. Each point is the average of main blood vessels in 4 to 5 CAMs (EA 6 to 7). The scale of time is asymmetric. The first 9 data points were recorded at 10-s intervals, and the next 10 were recorded at 3-min intervals.

The results show that the optical clearing agents reduce the flow velocity and influence the vessel diameter. The effects not only depend on the dosage including volume and concentration, but also on the kind of agent, glycerol or glucose. Glycerol induces a sharp decrease of blood flow velocity, i.e., 40 μL of 100% glycerol stops blood blow and block the blood vessels after 70 s. 25% and 50% glycerol reduce the blood flow and the diameter of lumen of the vessel and reach a plateau after 1.5 min and 16.5 min, respectively. In contrast, 40 μL of 40% glucose causes a durative decrease in blood flow velocity and diameter of lumen of the vessels. After application of 20% glucose, the blood flow is reduced gradually. However, the vessels are constricted during the first 10 min and then reach a constant. The effects of 10 μL of agents are relatively weaker. The relative decreases in blood flow velocity range from 35% to 72% at 31.5 min. 100% and 50% glycerol lead to durative constriction, but 25% glycerol and 20% and 40% glucose lead to constriction of vessels over the first several minutes. And then the vessels begin to dilate, and even the diameter is larger than the native value. There are slight decreases in blood flow and vessel diameter after application of physiological saline. The maximal relative change in blood flow is 3% and that in vessel diameter is 3.5%, which are much less than that after application of glycerol or glucose.

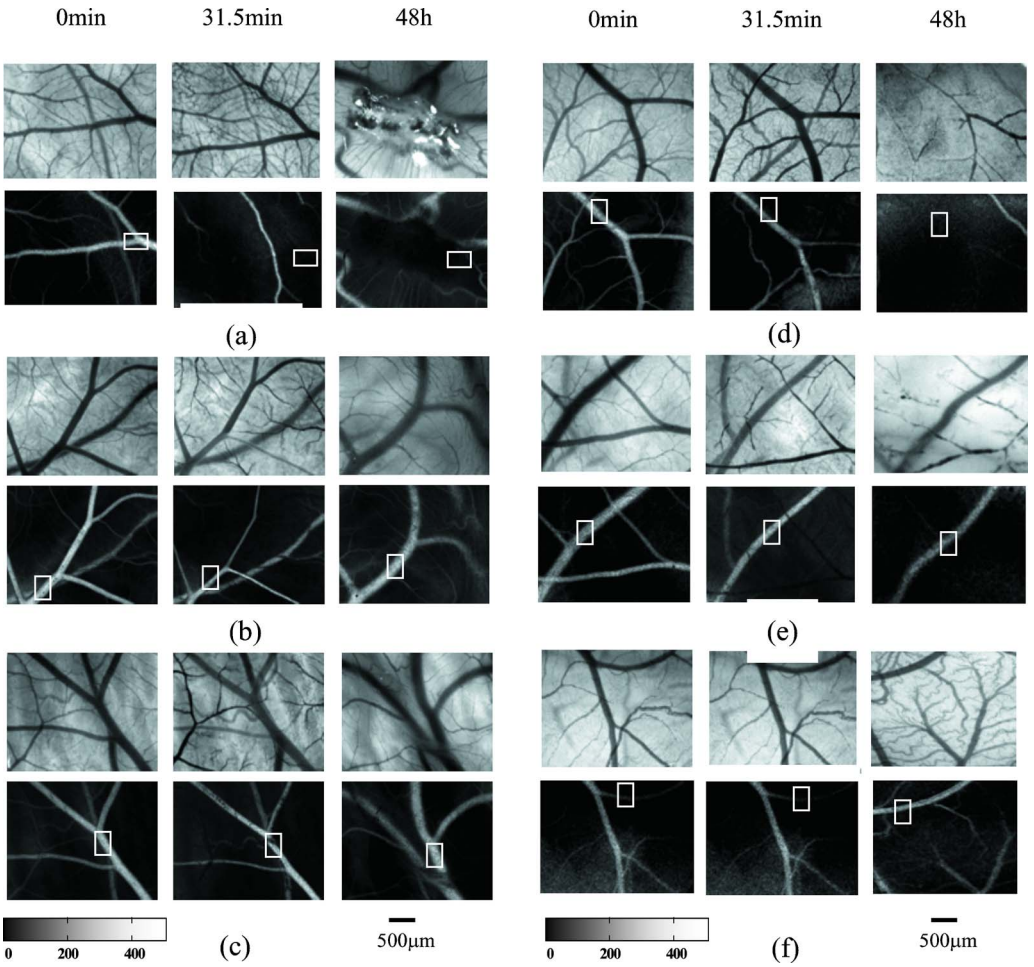

Fig. 4 Photographs and speckle velocity maps of blood vessels in CAM before, 31.5 min after and 2 days after application of different agents (40 μL): (a) 100% glycerol, (b) 50% glycerol, (c) 25% glycerol, (d) 40% glucose, (e) 20% glucose, and (f) physiological saline. In each subfigure, the top row is photographs, and the bottom row is speckle velocity maps. All of the imaging has the same gray scale and size scale. The gray scales below the images show the contrast scales, and the bar marks 500 μm.

3.2 *Long-Term Effects of Glycerol and Glucose on Blood Vessels*

Figures 4(a) to 4(f) show the typical photographs and the speckle velocity maps before and 31.5 min and 2 days after application of 40 μL of agents. In each subfigure, the top row is photographs, and the bottom row is speckle velocity maps.

In the control group, after 2 days, the diameter of the main blood vessels obviously increases, but only a part of the main vessels in the same region can be observed. In addition, many new blood vessels develop nearby [see Fig. 4(f)]. This means that the blood vessels of CAM can be well capable of growth. It should be noted that there are changes in the field of view at different time points. On the one hand, the diameter of the blood vessels will increase with the development of the chick embryo. Some blood vessels grow out of the initial region of the ring because new blood vessels develop. On the other hand, the chick embryo includes a yolk sac membrane, an amniotic membrane, and a chorioallantoic membrane, and each membrane can move independently.[38] Therefore, it is difficult to attain the same viewing field, especially, for long-term observations.

From the photographs in Figs. 4(a) to 4(e), we can observe that the optical clearing of blood vessels is improved to different extents, and some blood vessels lying underneath membrane are visible at 31.5 min after application of optical clearing agents. This means that the agents can increase the optical clearance of the CAM.

Comparing the images of native and 31.5 min with those of 2 days, long-term effects on blood vessels are observed. The results show that 100% glycerol induced local blood coagulation, which may result from red blood cell aggregates at the blood stasis. However, some larger blood vessels occur in the region-of-interest surroundings. After application of 50% and 25% glycerol, the diameter of large vessels increases more markedly than that in the control. There are new vessels, but the vessels are fewer in number. The higher the concentration, the fewer the number of vessels. Even though 40% glucose does not induce blockage of the blood flow at 31.5 min, there is no blood flow in the region of interest at 2 days. Compared with 40% glucose, the damage induced by 20% glucose is relatively weak, but no new blood vessel develops there. There is still blood flow in the main blood ves-

Table 1 Diameter of blocked vessels after application of agents (10 μL).

	Diameter (μm)					
	Glycerol			Glucose		
Time	100%	50%	25%	40%	20%	Physiological saline
1 min	60±10	35±10	30±10	45±15	25±5	—
31.5 min	70±10	50±10	40±10	65±15	40±10	—
48 h	70±15	55±15	40±10	85±20	60±15	—

sels, and the flow velocity at 2 days is slower than that at 31.5 min.

3.3 *Glycerol- or Glucose-Induced Damage to Blood Vessels*

We have made a statistical analysis on blockage of blood flow in vessels caused by different agents. Tables 1 and 2 show the diameter of blocked vessels at three time points: 1 min, 31.5 min, and 2 days after application of 100%, 50%, and 25% glycerol; 40% and 20% glucose; and physiological saline. The volume of agents given is 10 μL and 40 μL. Each value is from the average of 4 to 5 native CAMs.

The results demonstrate that physiological saline does not block blood flow, but both glycerol and glucose do. At the same time point, the diameter of blocked vessels increases with the volume and concentration of optical clearing agents. 10 μL of glycerol or glucose cause blockage of only small vessel branches, and the diameter of damaged vessels is less than 85 μm. 40 μL of agents cause blockage of large vessels, and even 100% glycerol and 40% glucose damage the main vessels. In addition, we can observe that short-term effects of glucose induce blockage of only some small vessel branches, but long-term observations denote that larger vessels are damaged. In contrast, the effects of glycerol mainly produce during the initial period. This means that the damage rate of blood vessel caused by glycerol is very quick, and that damage caused by glucose is relatively slow but similarly serious.

4 Discussion

The preceding results show the short-term and long-term effects of glycerol or glucose on blood vessels. Not only qualitative description but also quantitative analysis of the relationship between the effects and dosages of agents has been obtained.

4.1 *Animal Model*

The CAM is a convenient model that has many advantages over other *in vivo* models for evaluating short-term and long-tern effects on blood vessels caused by optical clearing agents. First, the transparent CAM enables optical imaging to monitor the dynamic changes in blood vessels. Second, the developing vasculature under the membrane can be used to mimic the neovasculature *in vivo* and observe changes in morphology and function of vessels. Third, the CAM is a simple and inexpensive animal model commonly employed for long-term observations in *in vivo* experiments. In addition, after application of optical clearing agents, some blood vessels underneath the membrane become visible. Therefore, when we monitor the changes of blood vessels in the CAM, we also observed the optical clearing effect of other membranes.

4.2 *Image Method*

With LSCI, a real-time, noninvasive technique for visualizing capillary blood flow, one can monitor blood flow in skin, brain, and mesentery.[24,30] By employing LSCI, not only infor-

Table 2 Diameter of blocked vessels after application of agents (40 μL).

	Diameter (μm)					
	Glycerol			Glucose		
Time	100%	50%	25%	40%	20%	Physiological saline
1 min	110±20	80±20	55±10	85±15	40±10	—
31.5 min	165±25	90±20	65±15	95±20	60±15	—
48 h	195±30	125±25	70±20	180±25	95±25	—

mation about the blood flow, but also information about the capillary density and diameter of the lumen of the vessels can be obtained.[31] The lumen of the vessel, where blood flows, is different from the external blood vessel. Photographs can show where there are blood vessels, while speckle velocity maps can indicate whether there is blood flow. We observe that there is no obvious morphological change in blood vessels after application of glucose, but many blood vessels are blocked. Therefore, it is very significant to measure the blood flow and the diameter of lumen of the vessels for investigation of blood vessel function.

4.3 *Short-Term and Long-Term Effects*

Different optical clearing agents have different effects on blood vessels, including short-term and long-term effects. During the initial period, the effects of glycerol are very strong, while those of glucose are relatively slight. If the blood vessels are not blocked completely by glycerol, the flow velocity will be recovered. Meanwhile, some new blood vessels develop in the region of interest with the development of the chick embryo, but the number of blood vessels is fewer. Therefore, the development of blood vessels of the CAM is still influenced to different extents by glycerol. In contrast, the effects of glucose experience a long period with no new blood vessel in the region of interest after application of glucose (20%, 40%). Hence the further damage to blood vessels is more serious than that of glycerol. These effects decrease with the volume of glycerol or glucose.

After having observed the effects of 100% and 75% glycerol on blood vessels for 50 min or more than 1 h, Vargas et al. indicated that glycerol might induce coagulation of blood.[24] Their results are basically coincident with ours. However, they did not show long-term observations.

However, the observations of Galanzha et al. appear to be somewhat different from ours. They found that glycerol led to local hemolysis of vessels and thought that the effects of glycerol are relatively constant and local but that the action of glucose is associated with more dilation of vessels without blood hemolysis.[25] From the view of short-term and long-term effects, it is easy to understand that the effects of glucose appeared relatively slight, because they had observed for only several minutes, and short-term effects of glycerol are stronger than those of glucose. Therefore, short-term observations are not enough to reflect completely the full effects of optical clearing agents on blood vessels. Long-term effects will demonstrate whether the optical clearing agent is safe for humans.

4.4 *Dose of Optical Clearing Agents*

From the view of dose, different doses also result in different effects, i.e., the effects of 10 μL are much slighter than those of 40 μL. First, the 10 μL of agents was given in Galanzha et al.'s experiments. Second, glycerol is more viscous than glucose. If there is no ring in the region of interest, as in this work, glucose will flow away but glycerol will not. Therefore, the effects of glycerol are more local than those of glucose, and the real dose of glucose may be less in their work. In fact, we have also observed the dilatation of blood vessels after application of 10 μL of 20% glucose.

Vargas et al. thought that glycerol might help in the treatment of blood vessel diseases because glycerol can block blood vessels.[23] However, they did not mention the volume of agents. Our results show that 40 μL of glycerol can block local blood vessels very quickly, but 10 μL blocks only small vessels. Therefore, the investigation on dosage of agents is similarly important.

4.5 *Optical Clearance of Tissue and Disturbances of Blood Vessel Function*

Among the investigations on optical clearing agents, higher-concentrated glycerol or glucose has a great optical clearance of tissue. For instance, by using OCT, Vargas et al. observed that 100% and 75% glycerol help to improve visualization of *in vivo* subdermal blood vessels of hamster dorsal skin.[24] Galanzha et al. applied spectrometry to measure the back-reflectance spectra of skin after glycerol or glucose was injected into the dermal layer of skin of rat. They indicated that 75% glycerol or 40% glucose can decrease the reflectance of skin and last for a while, but less-concentrated glucose solutions (20% to 35%) induce slight decreases or no.[25]

However, our results indicate that 40 μL of 100% glycerol blocks the main blood vessels and causes enduring damage to the local blood vessels. 25% or 50% glycerol leads to the cessation of flow in vessel branches and checks the development of blood vessels in the CAMs to different extents. 40% glucose leads to damage of blood vessels in larger regions and stops the growth of blood vessels. Although the damage to blood vessels caused by 20% glucose is slighter than that by 40% glucose, the growth of blood vessels is still stunted seriously. The effects depend on the dosage of agents, including volume and concentration. The lower the dosage, the less serious the effects.

Consequently, when optical clearing agents are injected directly into the dermis to improve the optical clearance of skin, the effects of the agents should be noticed, because the agents maybe also disturb the blood vessels' function. In fact, topical action of the agent to the epidermis is also performed, and thus the dosage of agents reaching into the blood vessels may be less than by direct injection. The effects on blood vessels will be reduced with the dosage of agents, but the optical clearance of tissue may be decreased. Therefore, we pay attention not only to the optical clearance of tissue caused by optical clearing agents, but also to clinical safety. Both short-term and long-term effects of agents on blood vessels will help to evaluate whether one agent is safe.

5 Conclusions

In this work, not only short-term effects on morphology of blood vessels and blood flow velocity in the CAM but also long-term effects on blood vessels after application of 10 μL and 40 μL of 100%, 50%, and 25% glycerol or 40% and 20% glucose were obtained. Short-term observations show that the agents reduce the local blood flow velocity and constrict and even block vessels. Long-term observations indicate that the agents stunt the development of blood vessels in the CAMs. Short-term effects of glycerol are very strong and are confined to a local region. The blood flow can be recovered to different extents if the blood vessel is not blocked completely. In contrast, long-term effects of glucose are more serious than short-

term effects. 40 μL of 40% and 20% glucose stop the development of local blood vessels in the CAMs. The effects will be decreased with the dose of agents.

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References

1. V. V. Tuchin, I. L. Maksimova, D. A. Zimnyakov, I. L. Kon, A. H. Mavlutov, and A. A. Mishin, "Light propagation in tissues with controlled optical properties," *J. Biomed. Opt.* **2**, 401–417 (1997).
2. V. V. Tuchin, "Optical immersion as a new tool to control optical properties of tissues and blood," *Laser Phys.* **15**, 1109–1136 (2005).
3. V. V. Tuchin, "Optical clearing of tissues and blood using the immersion method," *J. Phys. D* **38**(15), 2497–2518 (2005).
4. R. K. Wang, A. V. Priezzhev, and S. Fantini, "Editorial: special issue in honour of Professor Valery V Tuchin's contribution to the field of biomedical optics," *J. Phys. D* **38**(15), Editorial (2005).
5. K. Moulton, F. Lovell, E. Williams, P. Ryan, D. C. Lay Jr., D. Jansen, and S. Willard, "Use of glycerol as an optical clearing agent for enhancing photonic transference and detection of salmonella typhimurium through porcine skin," *J. Biomed. Opt.* **11**, 054027 (2006).
6. G. Vargas, K. F. Chan, S. L. Thomsen, and A. J. Welch, "Use of osmotically active agents to alter optical properties of tissue: effects on the detected fluorescence signal measured through skin," *Lasers Surg. Med.* **29**, 213–220 (2001).
7. R. Cicchi, F. S. Pavone, D. Massi, and D. D. Sampson, "Contrast and depth enhancement in two-photon microscopy of human skin *ex vivo* by use of optical clearing agents," *Opt. Express* **13**, 2337–2344 (2005).
8. S. Plotnikov, V. Juneja, A. B. Isaacson, W. A. Mohler, and P. J. Campagnola, "Optical clearing for improved contrast in second harmonic generation imaging of skeletal muscle," *Biophys. J.* **90**(1), 328–339 (2006).
9. M. H. Khan, S. Chess, B. Choi, K. M. Kelly, and J. S. Nelson, "Can topically applied optical clearing agents increase the epidermal damage threshold and enhance therapeutic efficacy?" *Lasers Surg. Med.* **35**, 93–95 (2004).
10. M. H. Khan, B. Choi, S. Chess, K. M. Kelly, J. McCullough, and J. S. Nelson, "Optical clearing of *in vivo* human skin: implications for light-based diagnostic imaging and therapeutics," *Lasers Surg. Med.* **34**, 83–85 (2004).
11. V. V. Tuchin, G. B. Altshuler, A. A. Gavrilova, A. B. Pravdin, D. Tabatadze, J. Childs, and I. V. Yaroslavsky, "Optical clearing of skin using flashlamp-induced enhancement of epidermal permeability," *Lasers Surg. Med.* **38**, 824–836 (2006).
12. O. Stummp, A. J. Welch, T. E. Milner, and J. Neev, "Enhancement of transepidermal skin clearing agent delivery using a 980-nm diode laser," *Lasers Surg. Med.* **37**, 278–285 (2005).
13. O. Stummp, B. Chen, and A. J. Welch, "Using sandpaper for noninvasive transepidermal optical skin clearing agent delivery," *J. Biomed. Opt.* **11**, 041118 (2006).
14. R. K. Wang, X. Xu, and V. V. Tuchin, "Concurrent enhancement of imaging depth and contrast for optical coherence tomography by hyperosmotic agents," *J. Opt. Soc. Am. B* **18**, 948–953 (2001).
15. R. K. Wang, X. Xu, Y. He, and J. B. Elder, "Investigation of optical clearing of gastric tissue immersed with hyperosmotic agents," *IEEE J. Sel. Top. Quantum Electron.* **9**, 234–242 (2003).
16. Y. He, R. K. Wang, and D. Xing, "Enhanced sensitivity and spatial resolution for *in vivo* imaging with low-level light-emitting probes by use of biocompatible chemical agents," *Opt. Lett.* **28**(21), 2076–2078 (2003).
17. X. Xu and R. K. Wang, "Synergetic effect of hyperosmotic agents of dimethyl sulfoxide and glycerol on optical clearing of gastric tissue studied with near infrared spectroscopy," *Phys. Med. Biol.* **49**, 457–468 (2004).
18. X. Xu, R. K. Wang, J. B. Elder, and V. V. Tuchin, "Effect of dextran-induced changes in refractive index and aggregation on optical properties of whole blood," *Phys. Med. Biol.* **48**, 1205–1221 (2003).
19. J. Hirshburg, B. Choi, J. S. Nelson, and A. T. Yeh, "Collagen solubility correlates with skin optical clearing," *J. Biomed. Opt.* **11**, 040501 (2006).
20. J. Hirshburg, B. Choi, J. S. Nelson, and A. T. Yeh, "Correlation between collagen solubility and skin optical clearing using sugars," *Lasers Surg. Med.* **39**(2), 140–144 (2007).
21. A. T. Yeh and J. Hirshburg, "Molecular interactions of exogenous chemical agents with collagen—implications for tissue optical clearing," *J. Biomed. Opt.* **11**, 0140003 (2006).
22. X. Xu and R. K. Wang, "The role of water desorption on optical clearing of biotissue: studied with near infrared reflectance spectroscopy," *Med. Phys.* **30**, 1246–1253 (2003).
23. H. Cheng, Q. Luo, S. Zeng, S. Cheng, W. H. Luo, and H. Gong, "Hyperosmotic chemical agent's effect on *in vivo* cerebral blood flow revealed by laser speckle," *Appl. Opt.* **43**, 5772–5777 (2004).
24. G. Vargas, A. Readinger, S. S. Dozier, and A. J. Welch, "Morphological changes in blood vessels produced by hyperosmotic agents and measured by optical coherence tomography," *Photochem. Photobiol.* **77**(5), 541–549 (2003).
25. E. I. Galanzha, V. V. Tuchin, A. V. Solovieva, T. V. Stepanova, Q. Luo, and H. Cheng, "Skin backreflectance and microvascular system functioning at the action of osmotic agents," *J. Phys. D* **36**, 1739–1746 (2003).
26. D. Knighton, D. Ausprunk, D. Tapper, and J. Folkman, "Avascular and vascular phases of tumor growth in the chick embryo," *Br. J. Cancer* **35**, 347–356 (1977).
27. S. Kimel, L. O. Svaasand, M. Hammer-Wilson, V. Gottfried, and M. W. Berns, "The chick chorioallantoic membrane for the study of synergistic effects of hyperthermia and photodynamic therapy," *Proc. SPIE* **1525**, 341–350 (1991).
28. Q. Liu, S. Zhou, Z. Zhang, and Q. Luo, "Laser speckle contrast imaging: monitoring blood flow dynamics and vascular structure of photodynamic therapy," *Proc. SPIE* **5630**, 26–33 (2005).
29. S. A. Telenkov, D. J. Smithies, D. M. Goodman, B. S. Tanenbaum, J. S. Nelson, and T. E. Milner, "Infrared imaging of *in vivo* microvasculature following pulsed laser irradiation," *J. Biomed. Opt.* **3**(4), 391–395 (1998).
30. H. Cheng, Q. Luo, S. Zeng, S. Chen, J. Cen, and H. Gong, "Modified laser speckle imaging method with improved spatial resolution," *J. Biomed. Opt.* **8**(3), 559–564 (2003).
31. D. Zhu, W. Lu, Y. Weng, H. Cui, and Q. Luo, "Monitoring thermal induced changes in tumor blood flow and micro-vessels with laser speckle contrast imaging," *Appl. Opt.* **46**(10), 1911–1917 (2007).
32. J. D. Briers, "Laser Doppler, speckle, and related techniques for blood perfusion mapping and imaging," *Physiol. Meas* **22**, R35–R66 (2001).
33. A. F. Fercher and J. D. Briers, "Flow visualization by means of single-exposure speckle photography," *Opt. Commun.* **37**, 326–329 (1981).
34. J. D. Briers, G. Richards, and X. W. He, "Capillary blood flow monitoring using laser speckle contrast analysis (LASCA)," *J. Biomed. Opt.* **4**(1), 164–175 (1999).
35. C. Cheng, C. Liu, N. Zhang, T. Jia, R. Li, and Z. Xu, "Absolute measurement of roughness and lateral-correlation length of random surfaces by use of the simplified model of image-speckle contrast," *Appl. Opt.* **41**, 4148–4156 (2002).
36. H. T. Yura and S. G. Hanson, "Comment: absolute measurement of roughness and lateral-correlation length of random surfaces by use of the simplified model of image-speckle contrast," *Appl. Opt.* **42**, 2521–2522 (2003).
37. C. Cheng, C. Liu, N. Zhang, T. Jia, R. Li, and Z. Xu, "Reply to comment: absolute measurement of roughness and lateral-correlation length of random surfaces by use of the simplified model of image-speckle contrast," *Appl. Opt.* **42**, 2523–2525 (2003).
38. G. Wang, C. Yi, and H. Zhou, "A technique of shell-less cultivation on chicken embryos for cancer microcirculation research," *Chinese J. Micro.* **12**(3), 32–34 (2002).